LIPID ACCEPTOR IN UDPGLUCURONIC ACID METABOLISM IN RAT LIVER MICROSOMES

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1. Introduction

Recent studies [1–6] have provided evidence that several monosaccharides in glycoprotein synthesis are bound to endogenous lipid acceptors. In the case of glucose the microsomal enzymes of the liver catalyze the transfer of the monosaccharide from UDPglucose to dolichol monophosphate [1–3]. Behrens et al. [3] also tried UDPglucuronic acid (UDPGlcUA) as donor, but found very little incorporation with natural dolichol monophosphate and none with synthetic polyprenol. Winsnes [7] failed to find a GlcUA carrier in the glucuronidation of 2-aminophenol. It has been suggested [8, 9] that DUPglucuronosyltransferase route associated with an endogenous glucuronide formation might play a role in the production of free GlcUA. This kind of endogenous GlcUA acceptor has not been isolated. UDPGlcUA can alos be converted to free GlcUA in mammalian liver via stepwise hydrolysis by a pyrophosphatase and a phosphatase, or via biosynthesis and hydrolysis of mucopolysaccharides [10, 11].

In this work we have found an endogenous lipid GlcUA acceptor and preliminarily studied its role in glucuronide biosynthesis and the formation of free GlcUA in rat liver microsomes.

Abbreviations: UDPGlcUA = Uridine-5′-diphosphate glucuronic acid; GlcUA-1-P = α-D-glucuronic acid 1-phosphate; GlcUA = D-glucuronic acid; GlcUAL = D-glucurono-6,3-lactone; 4-NO_2Phe-GlcUA = 4-nitrophenyl glucuronide (4-nitrophenol has been used as an exogenous GlcUA acceptor), X-GlcUA = an unknown glucuronide.

2. Materials and methods

2.1. *Preparation of microsomes, mitochondria, red blood cell ghost and rough lipid material*

Male 3–4 months-old rats fed ad libitum were used for the enzyme preparation. Older male and female rats were also used in the lipid extractions. The rats (*Rattus norvegicus*) were purchased as specific patogen free Wistar/Af/Han/Mol/(Han 67) and represent the second generation, outbred by the rotational mating system, in the Laboratory Animal Center of Kuopio University. Microsomes were prepared as previously described [12]. The microsomal pellet was resuspended into 0.15 M KCl to give about 30 mg protein/ml. The protein was determined by the Folin–Ciocalteau method [13]. This microsomal suspension was used in the enzyme assays.

The mitochondrial fraction was separated from rat liver as described earlier [14]. The mitochondria were washed twice by resedimenting, and then dried in a 1.3 kPa vacuum at room temperature.

The human red blood cell ghosts were prepared according to Hoffman [15] and dried as described above.

A rough lipid material was extracted from vacuum dried microsomes, mitochondria or red blood cell ghosts (0.1–1 g) three or four times with 10 ml of chloroform–methanol (2:1), acetone ethyl acetate or diethyl ether by mixing with Ultra Turrax 1 min at room temperature. In the case of acetone fresh microsomes were also used. After centrifugation for 10 min at 10 000 *g*, the combined supernatants were evaporated in an air stream. The residue was dissolved in a small amount of the same solvent and transferred to preweighted test tubes and evaporated in vacuo

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(1.3 kPa). The lyophylisation of the microsomal fraction or the evaporation of the solvent in a nitrogen stream did not increase the acceptor activity of the crude lipid material.

2.2. *Chemicals and enzymes*

Radioactive UDPGlcUA (^{14}C in GlcUA residue, specific activity > 200 mCi/mmole) was obtained from NEN Chemicals GmbH. Unlabeled UDPGlcUA (99% triammonium salt), α-D-glucuronic acid 1-phosphate (GlcUA-1-P), D-glucurono-6,3-lactone (GlcUAL) D-glucaro-1,4-lactone, phosphatidyl choline, phosphatidyl ethanolamine and testosterone were purchased from Sigma Chemical Co.. 4-Nitrophenol and K_2EDTA were purchased from Fluka AG. 4-Nitrophenyl-β-D-glucuronide, progesterone, tetrahydrocortisol and vitamin A alcohol were from Koch–Light Laboratories Ltd.. Synthetic dolichol monophosphate was prepared according to Behrens and Leloir [1]. Cholesterol monophosphate was synthesized as described by Montgomery et al. [16]. Other chemicals, not mentioned here, were the purest reagent grade (usually Merck pa. products).

Phosphodiesterase (beef heart), alkaline phosphatase (*E. coli*) and β-glucuronidase/aryl sulfatase (*Helix pomatia*) were obtained from Boehringer GmbH. Phosphatidases A (*Vipera russeli*), C (*Clostridium welchii*) and D (cabbage) were purchased from Sigma Chemical Co.

2.3. *Chromatographic procedures*

The metabolites and reference compounds were separated on Whatman No. 1 paper usually by descending chromatography. The following solvents were used: A = ethyl acetate–acetic acid–water (6:3:4); B = 2-propanol–ethanol–water (1:1:1); C = 1-propanol–methyl benzoate–acetic acid–water (5:2:2:3.5) [17]; D - toluene–1-butanol–acetic acid–water (7.5:2.5:3:7); E = ethyl acetate–1-hexane–acetic acid–water (6:4:1:9); F = 1-butyl acetate–1-butanol–acetic acid–water (8:2:1:9). The upper phases of solvents D–F [18] were used.

The unknown lipid–GlcUA compound was partly purified by paper chromatography. After separation from other metabolites with solvent A, the conjugate was eluted with the same solvent. The solvent was evaporated in vacuo.

2.4. *Determination of the acceptor activity*

Incubations were carried out over different time periods at 38°C in a shaking water bath (60 oscillations per min). The reaction mixture final volume 80 μl contained 0.4 M potassium phosphate (pH 7.5 except in pH optimum studies), 10 mM K_2EDTA, approx. 60 000 cpm [^{14}C] UDPGlcUA, variable amounts of rough lipid material or reference compounds studied as acceptor, 0.5% Triton X-100 (to solubilize the lipid material) and microsomes (about 0.6 mg protein). If the transfer of GlcUA from UDPGlcUA or an unknown lipid–GlcUA conjugate to 4-nitrophenol was studied, the reaction mixture also contained 0.5 mM of this exogenous aglycone.

Preincubations of a rough lipid material with alkaline phosphatase (350 mU), phosphodiesterase (10 mU), phosphatidases A, C and D (150, 500 and 500 mU respectively) were carried out in the buffer described above at 38°C. Labeled UDPGlcUA and microsomes were added 45 min later, and incubations were continued further for 30 min.

The incubations were terminated by adding 1.5 ml of chloroform–methanol (2:1), and the mixtures were prepared according to Folch et al. [19]. The aqueous phases were added to the denatured microsomal pellet, which was homogenized with a glass rod. After centrifugation aqueous or lipid phases were analyzed by paper chromatography.

Aliquots of the samples (30 μl) were applied on chromatographic papers and developed for 12 hr with solvent A using descending chromatography. The intermediates were located by staining the reference compounds. UDPGlcUA and GlcUA-1-P were visualized with molybdate–perchloric acid reagent [20] and GlcUA, GlcUAL and 4-nitrophenyl glucuronide with alkaline silver nitrate [21]. UDPGlcUA and 4-nitrophenyl glucuronide can also be located in ultraviolet light. The only radioactive compound in the organic phase after incubation was the unknown GlcUA–acceptor conjugate (X-GlcUA) (R_f 0.90 with solvent A), but most of it was found in the aqueous phase. The activities were measured from pieces of paper using a scintillation liquid containing 4.0 g PPO and 0.1 g POPOP in one litre of toluene with Wallac NTL 81 000 liquid scintillation counter.

The acetylation of the crude lipid material was carried out in a dry pyridine–acetic anhydride (3:1) mixture. Aliquots (dry weight 5–10 mg) of the lipid

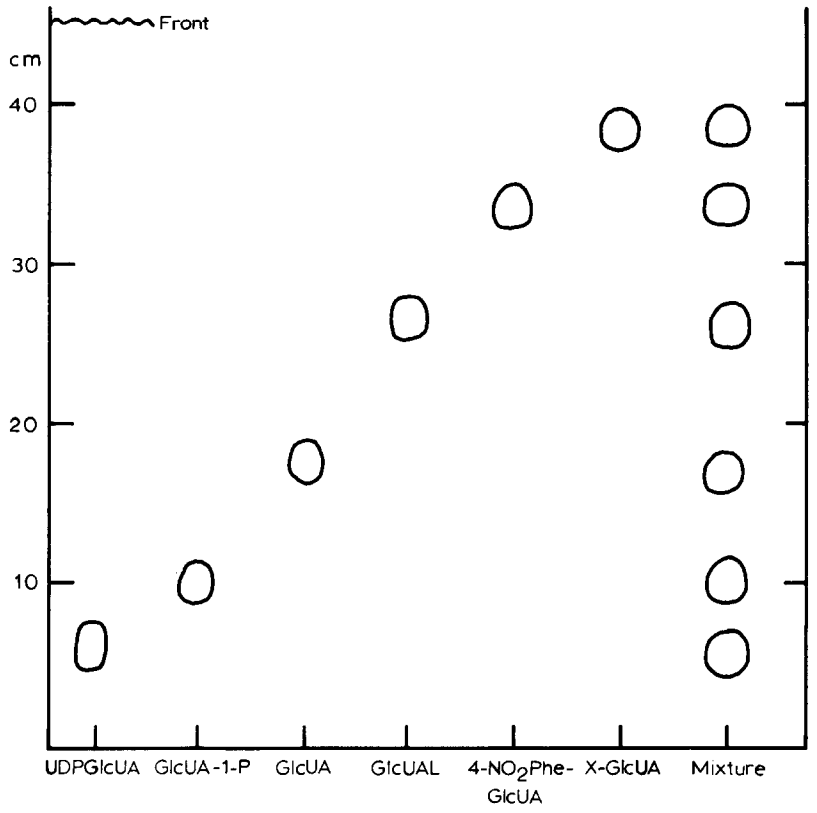

Fig. 1. Separation of the intermediates of UDPGlcUA in liver microsomes by descending 12 hr chromatography on Whatman No 1 paper with solvent ethyl acetate–acetic acid–water (6:3:4).

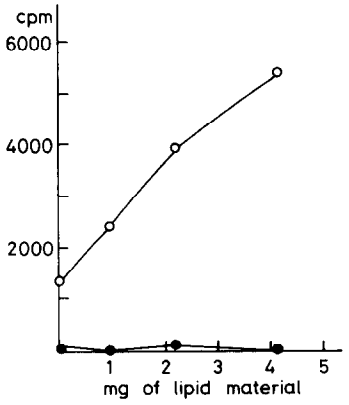

Fig. 2. Production of labeled material, soluble in the organic phase (Folch et al. [19] during incubation of the crude lipid material and [^{14}C] UDPGlcUA for 30 min at 38°C with (○–○) and without (●–●) native microsomes.

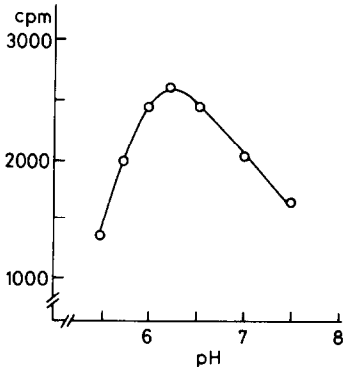

Fig. 3. Effect of pH on the incorporation of labeled GlcUA from UDPGlcUA into the crude lipid material with native microsomes. The relative activity was measured as cpm of X-GlcUA.

material were dissolved in 200 μl of mixture, and the tubes were kept for 45 min in a boiling water bath. The solvents were evaporated in vacuo.

3. Results

The intermediates of UDPGlcUA metabolism in rat liver microsomes could be separated by a descending paper chromatography for 12 hr with solvent A (fig. 1).

The liver microsomal and mitochondrial fractions and human red blood cell ghosts contained a lipid material which accepted GlcUA residue from UDPGlcUA during incubation with native rat liver microsomes. The acceptor could be extracted by chloroform–methanol (2:1), but it had almost the same solubility in wet and dry acetone, ethyl acetate and diethyl ether. The production of GlcUA conjugate of the lipid acceptor increased almost linearly with the amount of crude lipid material. No incorporation tookplace without native microsomes (fig. 2). X-GlcUA produced only one spot in six paper chromatographic solvents (A–F). The enzyme preparation was active in a wide range of pH with the optimum pH being 6.2 (fig. 3). Preincubations of the rough lipid material with alkaline phosphatase, phosphodiesterase, phosphatidases A, C or D did not reduce the acceptor activity. In the case of phosphodiesterase and phosphatidases C and D a slight activation in the production of X-GlcUA was found. Acetylation of the lipid material decreased the acceptor activity by about 50%. The increase in the acetylation time (up to 90 min) or the relative amount of acetylation reagents did not further diminish the acceptor activity.

The crude lipid material was found to activate UDPglucuronosyl-transferase when 4-nitrophenol was used as aglycone (fig. 4). 4-Nitrophenyl glucuronide was not produced when partly purified X-GlcUA and 4-nitrophenol were incubated with native microsomes in 0.5 M phosphate buffer (pH 7.5) containing 2 mM $MgCl_2$ and 0.5% Triton X-100. The addition of 4-nitrophenol to the reaction mixture did not affect the X-GlcUA production, but in higher concentrations the lipid material reduced the conjugated of this exogenous aglycone.

The GlcUA containing conjugate was hydrolyzed by microsomal and *Helix pomatia* β-glucoronidase to

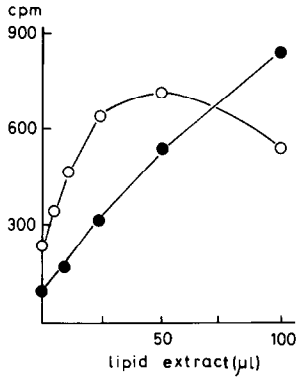

Fig. 4. Effect of chloroform–methanol (2:1) extract on the production of radioactive 4-nitrophenyl glucuronide (○–○) and X-GlcUA (●–●) during incubation of the extract, [^{14}C] UDPGlcUA, 4-nitrophenol and microsomes. The solvent from the extract was evaporated before incubation. The sample for paper chromatography was taken from the water phase (Folch et al. [19]).

a substance which had the same velocity as GlcUA in three different paper chromatographic solvents (A, B and C). D-Glucaro-1,4-lactone powerfully inhibited the hydrolysis, and the addition of this inhibitor to the reaction mixture increased the yield of X-GlcUA during incubation.

Several standard compounds were studied as GlcUA acceptors including phospholipids (synthetic dolichol monophosphate, cholesterol monophosphate, phosphatidyl choline, phosphatidyl ethanolamine) fat soluble vitamins (vitamin K_3, vitamin A alcohol) steroid hormones (progesterone, testosterone, estriol, tetrahydrocortisol) cholesterol and bilirubin. Either the compounds studied accepted no GlcUA under the reaction conditions used or their conjugates had R_f values differing from those of X-GlcUA.

4. Discussion

The results indicate that there is a lipid GlcUA acceptor in liver microsomes, mitochondria and red blood cell ghosts. GlcUA residue from UDPGlcUA is transferred to the compound in a reaction catalyzed by microsomal enzyme(s). X-GlcUA seems to be homogenous, because it produced only one spot in six different paper chromatographic solvent systems. Because preincubation of a crude acceptor lipid with

different types of enzymes hydrolysing phosphate esters did not decrease the acceptor activity, it seems improbable that compound is a phospholipid. In good agreement with this are the results that the acceptor could also be dissolved in diethyl ether and acetone, which are unable to dissolve phospholipids. A slight elevation in the acceptor activity after preincubation with phosphodiesterase and phosphatidases C and D might be produced by an activation of microsomal enzymes (for instance UDPglucuronosyl-transferase [22, 23] is activated by phosphatidase C). The increment of the acceptor activity might also be produced by the better solubility of an acceptor after prein cubation with the enzymes mentioned above.

The GlcUA conjugate produced during incubation seems to be β-glucuronide, because free GlcUA could be released from it by β-glucuronidase and the specific inhibitor, D-glucaro-1,4-lactone, blocked the hydrolysis. At least part of the GlcUA is conjugated to the acceptor molecule by hydroxyl or amino group(s) since acetylation of a crude lipid material with pyridine–acetic anhydride mixture reduced the incorporation. The effect of the acetylation on the acceptor activity may be more drastic than found, if part of the acetylated acceptor is hydrolyzed by microsomal esterase(s) during incubation with UDPGlcUA, and thus again might be able to conjugate with GlcUA.

Even if the crude lipid material increased the glucuronidation of an exogenous aglycone, 4-nitrophenol, the lipid acceptor probably does not play a role as an intermediate carrier in glucuronidation reactions as does dolichol monophosphate in glycoprotein synthesis [1, 2]. We were not able to synthetize any 4-nitrophenyl glucuronide by microsomes when partly purified X-GlcUA was a GlcUA donor and 4-nitrophenol an acceptor. The activation effect of a rough lipid material on the glucuronidation seems to be caused by other components in the extract. For instance some fatty acids, lecithin and lysolecithin have been reported to activate UDPglucuronosyltransferase [23, 24]. X-GlcUA could, however, be hydroluzed by microsomal β-glucuronidase and thus be a source of the biosynthesis of free GlcUA.

Further identification of the acceptor molecule and studies on its role in UDPGlcUA metabolism are under way in our laboratory.

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